Investigation of Volatile Metabolites in Sebum as Prodromal Indicators of Parkinson's Disease

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Abstract

Background: Parkinson's Disease (PD) has been associated with a distinct odour, strongest in sebum-rich areas. Thermal Desorption – Gas Chromatography – Mass Spectrometry (TD-GC-MS) has revealed volatile signatures that distinguish individuals with Parkinson's Disease (PD) from healthy controls. Here, we applied the same method, including subjects with isolated REM sleep behaviour disorder (iRBD) to examine the volatiles in sebum and compare this with that found in PD subjects and control participants. Participants with iRBD have a high likelihood for conversion to overt clinical synucleinopathies like PD, Dementia with Lewy Bodies (DLB) or (less commonly) Multiple System Atrophy (MSA).

Methods: Subjects with clinically established PD (n=16) or iRBD (n=9) as well as healthy controls (n=9) were included. Following methods established in our laboratory, sebum was sampled from each participant using cotton gauze and the headspace from these swabs, analysed directly with TD-GC-MS[1,2]. Univariate and multivariate analysis was employed to probe the differences between volatile metabolites found for each phenotype. Putative identifications were assigned using spectral matching against the Golm metabolome and NIST spectral databases.

Findings: We can completely distinguish each phenotype using top 25 significant molecules in the sampled volatilome. The classification between PD and control improved on previously published work, from 85% to 100%. Putatively annotated molecules include alkanes, aldehydes, fatty acid methyl esters (FAMEs), and three metabolites namely purine, tropinone and oleamide. Investigation of highly ranked features revealed 18 features that showed intermediate expression in samples from iRBD participants.

Interpretation: TD-GC-MS can differentiate volatile metabolite signatures from sebum between PD, RDB and control samples. More than 70% of the identifiable metabolites that

permit this discrimination were putatively annotated as hydrocarbons and fatty acid methyl esters (FAMEs). Our prior work indicates that these components arise from larger lipid molecules that decompose during the experiment[2]. Features putatively annotated as tropinone, oleamide and purine, have previously been linked with neuroprotection, sleep induction and antioxidation, respectively, are significantly different between the three groups of participants, along with FAMEs and hydrocarbons.

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Introduction

Parkinson's Disease (PD) is the second most common neurodegenerative disorder, with more than six million people affected worldwide and an expected two-fold increase in prevalence over the next generation[3]. The pathological hallmarks are neuronal α-synuclein positive inclusions and cell loss in the *substantia nigra,* other areas of the brain, and the peripheral autonomic nervous system causing the cardinal motor features of bradykinesia, rigidity and rest tremor as well as a plethora of non-motor symptoms[4]. Current diagnostic criteria for PD are anchored on clinical features, which overlap with other conditions and may result in initial misclassification[5]. Highly sensitive and specific tests, therefore, are a major need in PD - both to enhance diagnostic accuracy and to enable detection of the earliest disease stages where future disease-modifying therapies would be the most effective[5].

Clinically manifested PD evolves from a prodromal stage characterised by the occurrence of a variety of non-motor symptoms, including anosmia, constipation, anxiety, depression, change in cognitive behaviour as well as REM-Sleep Behaviour Disorder (RBD)[6,7]. Together with various risk markers, these have been used to define research criteria for prodromal PD. Within these criteria a polysomnographic diagnosis of isolated (iRBD), characterised by loss of REM-atonia and jerks or dream-enacting behaviours during REM sleep confers the highest risk for future PD with a likelihood ratio of 130[8]. Large prospective studies of cohorts with iRBD have shown conversion rates to clinically manifest synucleinopathies like PD, Dementia with Lewy Bodies (DLB) or Multiple System Atrophy (MSA) of more than 80% after follow-up for ten years or more[9]. Recent insights into prodromal features and risk markers for PD have led to the launch of population-based screening for PD risk, using multistep protocols where an initial online remote screen is followed by in-person tests, including dopamine transporter imaging[7].

Sebum is a lipid-rich substance secreted by the sebaceous glands on the skin, which are most concentrated on the face and upper back[10]. Increased sebum output (seborrhoea) and seborrheic dermatitis are well-known features of PD and changes in volatile profiles of sebum have recently been detected using Thermal Desorption – Gas Chromatography – Mass Spectrometry (TD-GC-MS)[1,2]. This method has so far not been employed on cohorts showing prodromal PD symptoms, which would be important to understand if volatile profiles of sebum have potential as biomarkers for early diagnosis. In this study, we analysed sebum samples from subjects with PSG-confirmed iRBD and compared findings to samples obtained from subjects with Parkinson's Disease (PD) and healthy controls in order to investigate differences in sebum composition and corresponding volatile metabolites profile. We describe how odour and mass spectrometry markers permit classification between the different phenotypes.

Odourous volatilome and metabolic markers can thus, be indicative of progression from iRBD to PD (Figure 1).

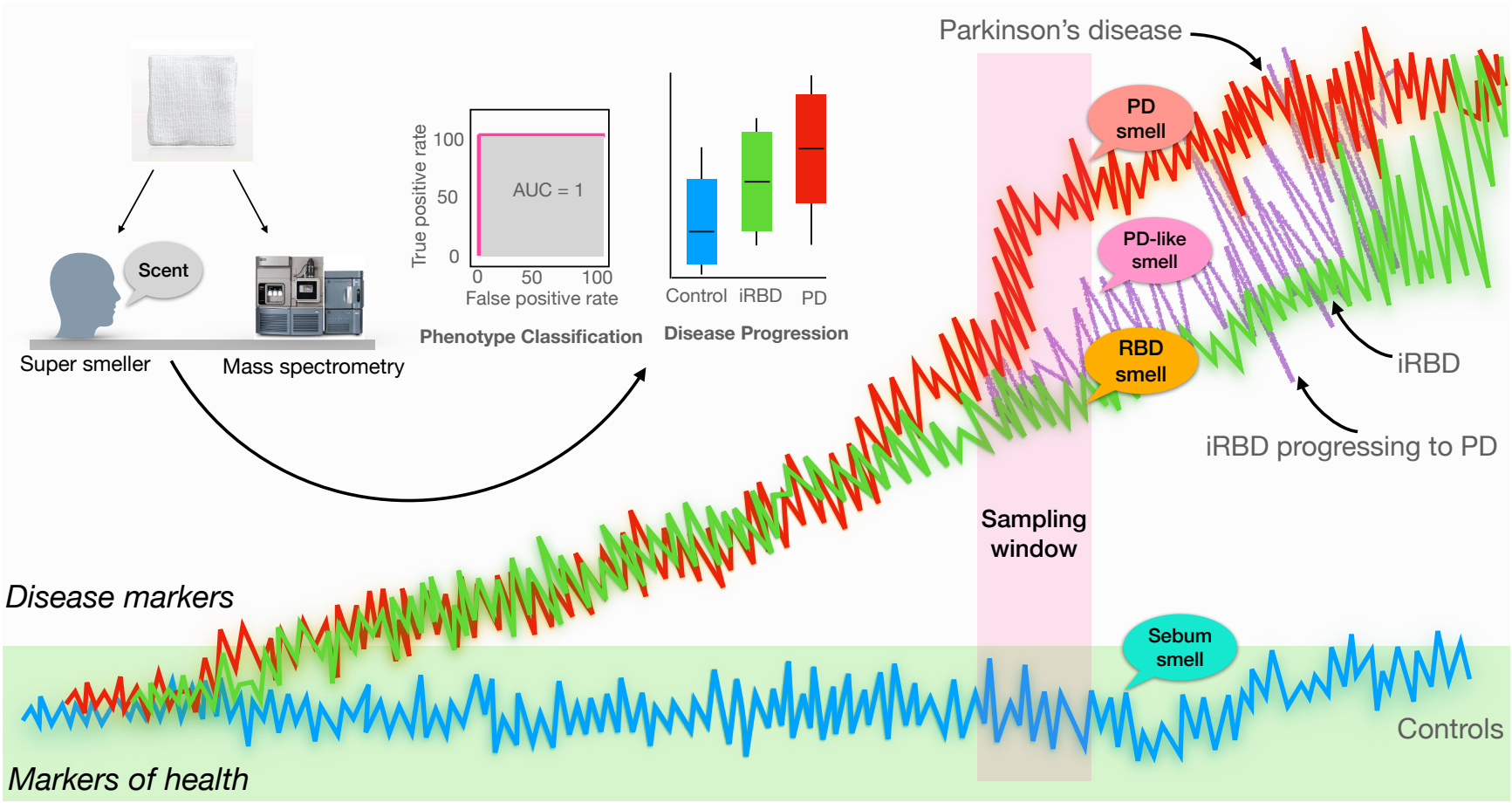

Figure 1: Schematic which shows the ability to distinguish a disease phenotype with different methods, namely, TD GC-MS and olfaction and here places this on a clinical assessment of the disease (depicted by the colour of the progressive biomarkers). The figure illustrates that we are sampling at a single time point that falls somewhere in this trajectory, and we used a window since the samples were not all taken at the same time.

Healthy sebum biomarkers (Blue) are distinguished from iRBD (Green) and PD (Red) using both mass spectrometry and olfactory analysis. Mass spectrometer data allows us to classify phenotype from variables and determine the extent of true positives (ROC curve). By examining expression change of phenotypic features, we can determine markers that show the change from healthy to diseased. In parallel we investigated the odour from the sebum of each participant group and determined that each disease has a distinctive smell above that of healthy sebum, and remarkably that the change in odour could also be a progression indicator.

Sample collection and clinical cohort

Subjects were recruited at the Movement Disorder and Sleep Disorder out-patient clinics of the Dept. of Neurology of the Medical University Innsbruck, Austria. Site staff, patients' caregivers, and patients without neurodegenerative or dermatologic disorders were invited to participate as healthy controls. The study was reviewed and approved by the local ethics committee (EK number AN1979d 336/4.19) and all participants provided written informed consent. A total of 34 subjects were included (16 PD, 9 iRBD and 9 controls) and participant demographics are detailed in Table 1. Two iRBD patients developed clinical signs of Parkinsonism within 12 months after sebum sampling.

All samples were collected by the same individual to limit alterations in technique. Samples were collected on cotton-based gauze swabs (7.5 x 7.5 cm) that was wiped firmly on the upper back of participants, following the sampling protocol as described in earlier work[1,2]. Samples were transferred to airtight bags where they were sealed and stored at -80˚C until analysis. After collection, samples were blinded and randomised for the analytical team at Manchester, UK until the data analysis and interpretation stage. Dynamic headspace was analysed from these samples in a randomised order. Raw data were deconvolved, annotated and converted to a data matrix consisting of 613 features and statistical interrogation of data was undertaken. The materials used, analytical method setup and data processing is described in supplementary information (S1, S2 and S3, respectively).

Table 1: Participants' characteristics at the time of recruitment. Nine PD participants and all iRBD participants had PSG recordings, with eight PD participants having PSG-confirmed iRBD. Two of the PSG-confirmed iRBD participants developed signs of Parkinsonism at clinical follow ups but did not show signs of Parkinsonism at the time of sampling. None of the controls showed any signs of Parkinsonism or clinical history of RBD at the time of sampling.

Phenotype	Age - mean (SD)	Sex (M: F)	BMI - mean (SD)	PSG confirmed RBD (*n*=)	Medication for PD (*n*=)	RBD-Medication (*n*=)
PD (*n*=16)	64.62 (6.38)	3:1	25.78 (3.78)	8	16 LDOPA: 14 DA: 11 MAO-BI: 9 amantadine: 5	4 clonazepam: 3 melatonin: 1
iRBD (*n*=9)	69.89 (11.83)	8:1	27.93 (4.69)	9	- DA agonists for RLS:1	3 clonazepam: 2 melatonin: 1
C (*n*=9)	60.44 (4.80)	8:1	27.06 (3.61)	0	-	-

C, controls; DA, dopamine agonist; LDOPA, Levodopa; MAO-BI, MAO-B inhibitor; PD, Parkinson's disease; RBD, REM sleep behavior disorder; RLS, restless legs syndrome.

Results and discussion

Classification of phenotypes

In order find features from the volatilome signature that differentiate between the three phenotypes, we used two approaches. We used ReliefF ranking algorithm and Kruskal-Wallis ANOVA (described in Supplementary Information). ReliefF estimates the quality of any feature in the context of other features and is non-parametric method. We investigated top 25 features as our features of interest (*FoI*). Out of these top 25 *FoI*, 19 were also significantly different ($p < 0.05$) after FDR correction for multiple testing problem. The presence of volatilome signature that significantly differentiates PD and control samples validate our previously published findings[1,2]. Figure 2A shows clear separation and complete classification of phenotypes using only *FoI.* Multivariate receiver operator characteristic (ROC) curves show that binary classifier built using *FoI*, can completely distinguishing PD participants from iRBD as well as controls. Even when using just five *FoI* we can achieve AUC of 90% and 100% when modelling for PD against controls and PD against iRBD respectively (Figure 2B).

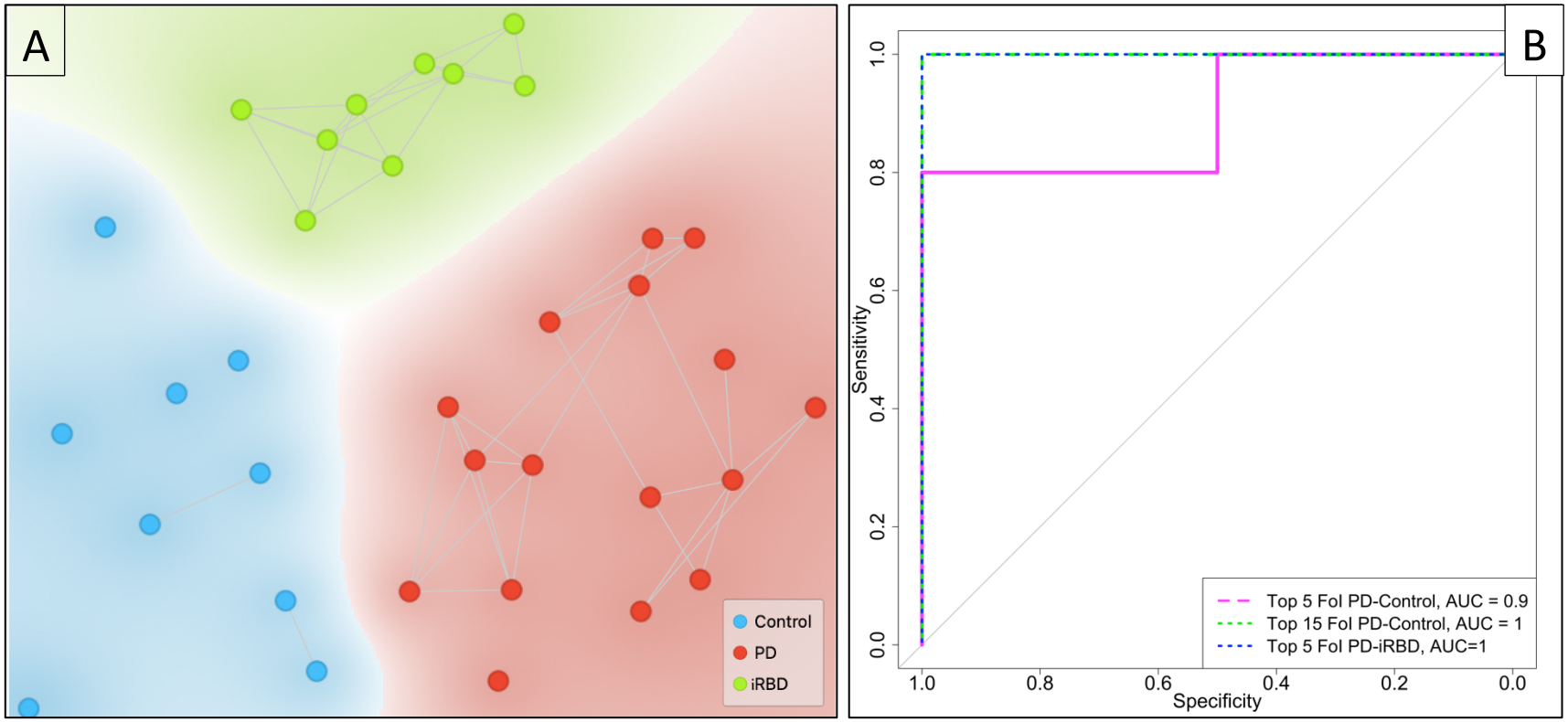

Figure 2: (A) *MDS plot showing clustering by phenotypes using FoI. Lines joining dots indicate which of the samples within groups were more similar than the rest, indicating common features in the disease phenotype and a far higher degree of dissimilarity in the control samples. (B) Multivariate ROC curve shows using the top five FoI, AUC=0.9 is obtained for PD vs Control classification. For both cases, using 15 or more FoIs (PD vs Control) and using five or more FoIs (PD vs iRBD) an AUC of 1 is obtained.*

Abundance of volatiles by phenotypes

Comparing means of these features across three phenotypes nine showed an increase from control to iRBD to PD and nine showed a decrease from control to iRBD to PD (Figure 2). These 18 features indicate iRBD as an intermediary phenotype between a control and a Parkinson's phenotype. There was one feature found to be highest in iRBD, six were lowest in iRBD (Supplementary S4). The *FoI* were putatively annotated as alkane (10), FAMEs (4), aldehyde (2), alkene (1), dialkyl ethers (1), purine (1) and tropinone (1). Four of the *FoI* could not be matched to library and thus, classed as unknown. Lack of identification of these species could be due to chromatographic co-elution, lack of precursor ions or lack of suitable match in the underivatized spectra in libraries – all preventative in assigning a biologically relevant annotation. Nonetheless, the complete classification of iRBD samples from control and PD, suggests that this disease has a distinct volatilome in terms of the abundance of identified features.

A sebum volatilome pattern of iRBD, that for most of its components, appears intermediate between that seen in controls and PD subjects is consistent with the notion of iRBD reflecting a prodromal state of PD. This consecutively lends further support to the sebum volatilome as a diagnostic biomarker for PD. This is best represented by viewing box plots of the identified *FoI* (Figure 3). We have plotted the *FoI* in terms of their different abundances in each participant group. In Figure 3A, we have shown compounds that increase in expression from control to PD, and in Figure 3B those that decrease from control to PD as well as some where the levels between PD and iRDB are not significantly different. It is unknown, if any of these are more sensitive than others to indicate phenoconversion from iRBD to PD or which of these changes earlier or after the phenoconversion. This will require further longitudinal studies. Also, since iRBD can evolve into Lewy Body disorders (PD and DLB) or MSA, the question remains which of the iRBD-associated features observed in this study are most

specific for conversion to either of these conditions. With our current sample size and lack of prospective data, it is not possible to comment if there are underlying volatilome signatures of iRBD (*i.e.*, within group variations or lack thereof seen in cluster analysis) that can be associated with conversion trajectory towards PD/DLB or MSA.

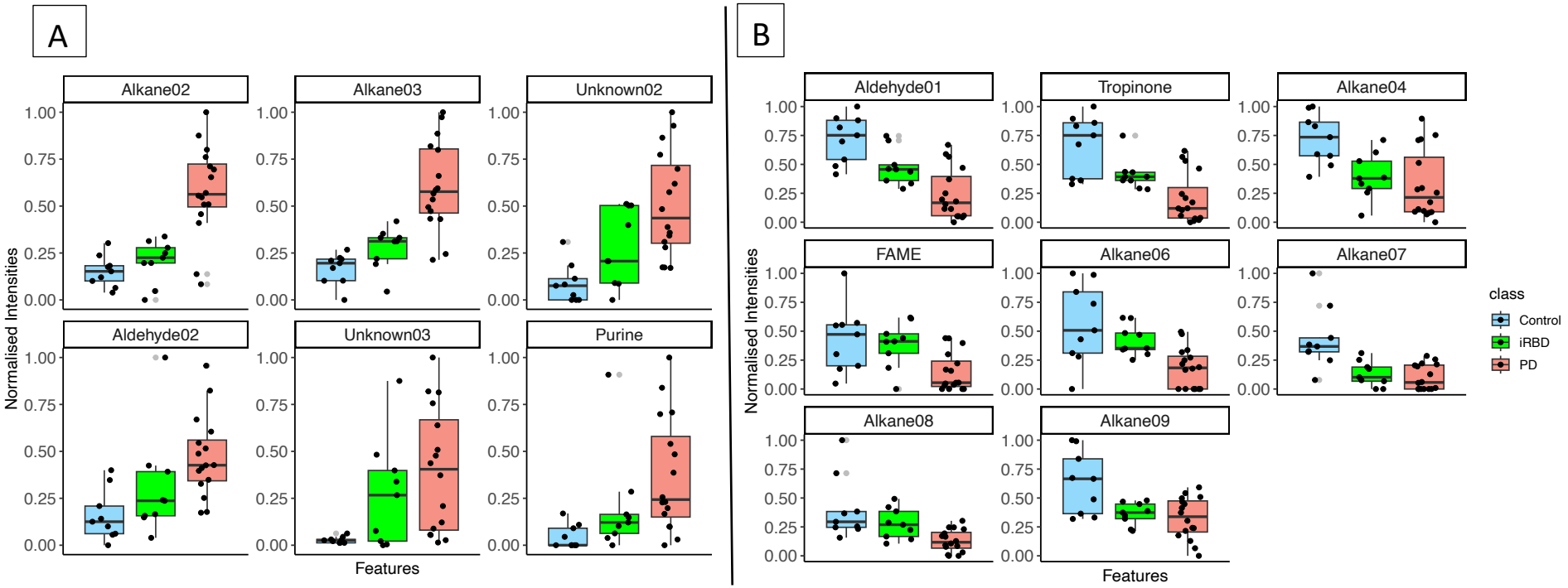

Figure 3: *Out of 25 FoI, 14 features had intermediate expression in the iRBD participants. These consisted of putatively annotated alkanes, aldehyde, FAME, tropinone and a purine species. Boxplots compare mean normalised intensities (black lines) between these phenotypes. Plots of features are ordered in their ranked importance. (A) compounds that increase in expression from control to PD (B) compounds that decrease from control to PD. All these compounds also had p-value of less than 0.01 after FDR correction. Each black dot is an individual sample and each grey dot represents an outlier.*

Interpretation of volatilome changes

Changes in fatty acid concentrations in brain tissue[11], blood[12] and the gut[13] have previously been associated with PD, and we have also reported alterations in lipids with fatty acid chains in sebum[14]. Analysing lipid standards with GC-MS gives rise to several resolvable features, identified as alkanes, as opposed to a single identifiable species as expected, which indicates that the ionisation process causes substantial fragmentation[2]. We hypothesise that the alkane *FoI* are derived from larger fatty acids and FAMEs in sebum. Fatty acids are a well-documented product of the breakdown of larger lipid molecules, and acylglycerols[15-17]. Similarly, aldehydes in headspace may arise due to oxidation of lipids, either endogenously or during the analysis[18].

Tropinone which was observed to decrease in iRBD and further decrease in PD, is an alkaloid derived from putrescine[19]. Studies in animal models have shown an important role of putrescine in skin physiology and neuroprotection[20]. Oleamide, an endogenous fatty acid was observed in the top 30 highly ranked features, and it had lower expression in the iRBD cohort compared to controls in this study. It is a sleep-inducing lipid, which has been found to decrease motility in animal models[21,22]. It reduces sleep apnoeic events and affects amount of REM and non-REM sleep in rat models[23,24]. Oleamide along with a few more alkanes and FAMEs were putatively annotated features that were not one of the *FoI*s due to lower ranking. Purine was observed to be lowest in control and highest in PD, with intermediate expression in iRBD. One of the end products of purine metabolism is urate - an antioxidant that is a risk marker for PD, and lower levels are also associated with higher severity of disease[25-27]. Alterations in the purine pathway have been researched in an investigation for PD biomarkers, as well as in comparison with other neurological dieases[28,29]. In iRBD, the intermediate expression of these molecules and breakdown products discussed earlier, compared to PD and controls may indicate purine and tropinone along with certain classes of lipids may be markers of prodromal PD.

Interpretation of volatilome changes with regards to body odour

When presented with PD, iRBD and control gauze, Joy Milne, with her hypersensitivity to smell due to hereditary hyperosmia[30], was able to describe the presence of three distinct odours from gauze. The smell of PD as described earlier was thick, musky, that of iRBD was biscuity sweet and scent of control swabs was described as a person odour lacking any of the other smells. In this blind exploration, Joy was able to hint at mosaic presence or mix of PD and iRBD scent in some of the iRBD participants. Out of the three iRBD participant samples, where hints of PD smell were described, two developed clinical signs of Parkinsonism within 12 months after sebum sampling. Based on these data, we further hypothesis that onset of Parkinsonism may alter body odour prior to detection by volatilome profiles measured by GC-MS or even current clinical symptoms (Figure 1) which implies a possibility of early detection of iRBD conversion to PD.

Limitations of the study

The key limitations of this study were smaller sample numbers which were unavoidable due to selective phenotypes and majority of participants were on medication as a treatment. The cohort was however, controlled for age and BMI to avoid confounding effects on the measurements. No strong associations were found between the *FoI* and known medications namely madopar, l-dopa, carbidopa, entacapone, ropinirole, rasagiline, apomorphine, amantadine, pramipexole, propranolol, clonazepame (rivotril), quetiapine, melotonin and rotigotine.

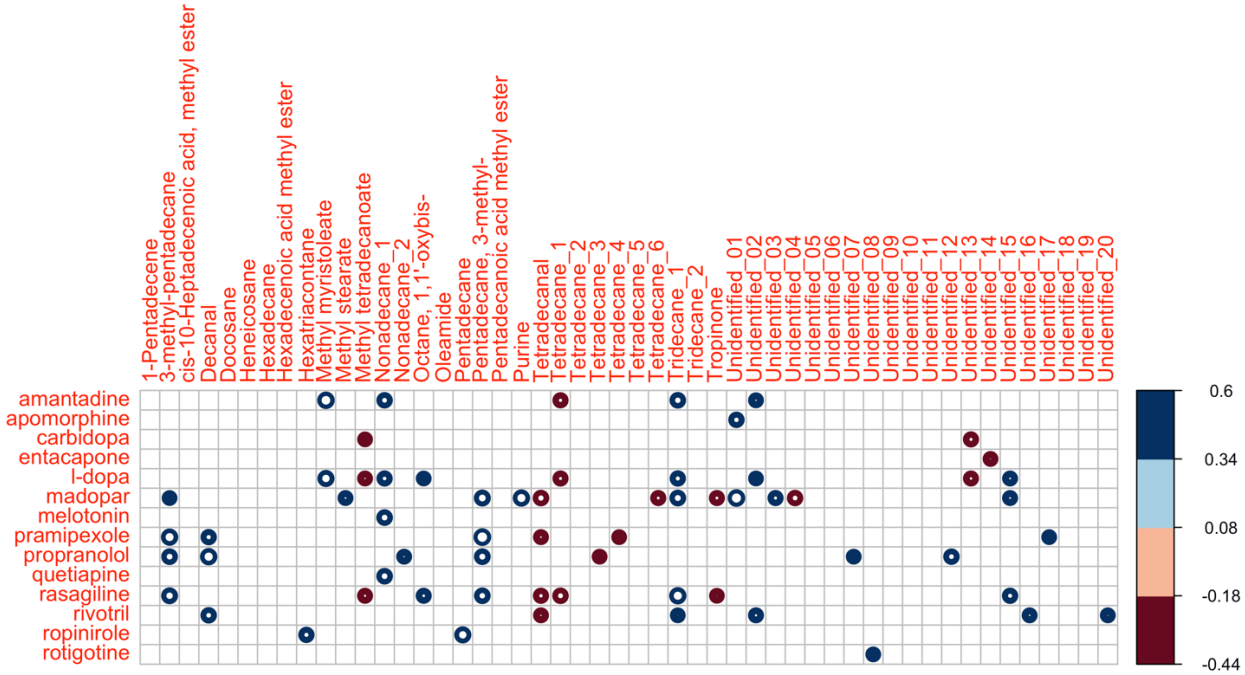

Figure 4: *Pearson's correlation analysis between drugs and top 50 ranked features suggests very weak correlation (less than 0.6 and greater than -0.4) between drug intake and measured intensities. Each row refers to medication and each column is one of the top 50 ranked features. The colour of circle represents value of coefficient correlation R. The diameter of hollow circle represents narrow difference between lower and higher bounds of 95% confidence intervals (CI). The circles with no hollow inner circle represent big difference in higher and lower end of CI.*

Although some associations were significant, they were not strong enough. This indicates the detected volatilome may not be contain directly measurable drug metabolites and other metabolites detected are not affected due to participant's medication history. Our previous work has shown that volatilome analysed with current method, is not associated with or confounded due to medication intake or dosage[2]. Due to lack of non-derivatised volatilome library that covers endogenous metabolites, it was not possible to assign definite annotation which prevented meaningful metabolic pathway enrichment analysis. However, the results indicate there is a clear, discriminating volatilome signature that is present on skin swabs collected from all three cohorts.

Conclusion

This experiment has shown distinct volatilome signatures detectable from sebum in Parkinson's disease, iRBD and control phenotypes. We have shown that we can classify with high certainty between these phenotypes using this volatilome signature. Upon further investigation of the significant features in volatilome, we have shown many metabolite signals to be the result of lipid degradation such as hydrocarbons and fatty acids. Thus, the detection of these maybe due to the alteration in lipid composition in sebum with the disease. The expression of some of these features in iRBD cohort is intermediate between that of PD and control suggesting that change in expression of certain lipids present in human sebum and metabolites such as alkaloids and purine could be early indicators of pre-manifest PD.

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